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I, DAVID DANIEL CLARKE , ASSISTANT DIRECTOR PATENT SERVICES, hereby certify that the annexed are true copies of the Provisional specification and drawing(s) as filed on 1 April 1996 in connection with Application No. PN 9047 for a patent by LIONS EYE INSTITUTE filed on 1 April 1996.

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WITNESS my hand this Twenty-Eighth day of October 1996

DAVID DANIEL CLARKE
ASSISTANT DIRECTOR PATENT SERVICES

AUSTRALIA

Patents Act 1990

PROVISIONAL SPECIFICATION

Applicant(s):

LIONS EYE INSTITUTE

Invention Title:

METHOD AND COMPOSITION FOR TREATMENT OF OCULAR DISEASES

The invention is described in the following statement:

METHOD AND COMPOSITION FOR TREATMENT OF OCULAR DISEASES

This invention relates to a method and composition for treating ocular diseases, in particular retinal disease involving neovascularisation of the choroid and/or retina. It makes use of the phagocytic characteristic of specific cells in the eye to provide an effective manner of delivering an active agent to the target, for either short term or long term treatment of neovascularisation. The methods and compositions of the invention are useful for delivering DNA, RNA, anti-sense nucleotides, peptides or other therapeutic agents to phagocytic cells or surrounding cells.

BACKGROUND OF THE INVENTION

A variety of ocular diseases such as macular degeneration and diabetic retinopathy are characterised by neovascularisation of the choroid and/or retina. This process is the major cause of blindness in patients suffering from these conditions.

PRIOR ART TREATMENTS

In age-related macular degeneration (ARMD), the formation and haemorrhaging of a subretinal neovascular membrane (SRNVM) results in rapid and substantial loss of central vision. Various treatments are available, but all are unreliable. Laser photocoagulation is the most acceptable type of treatment, but it still suffers from the disadvantages that damage by the laser rays causes dense, permanent scotoma (Schachet, 1994; Ibanez *et al*, 1995 and Hudson *et al*, 1995) resulting in temporary loss of vision, and inability to prevent progression of the condition in the long term because of recurrence of the neovascular membrane.

Thus this treatment provides an advantage only in terms of pr venting *profound* visual loss.

Similarly, surgical removal of the SRNVM or of subretinal blood, or r -positioning of the fovea by rotating the retina have largely been unsuccessful, due to post-surgical complications and to minimum or temporary improvement in vision. These invasive forms of treatment and the corresponding complications therefore far outweigh the advantages gained, and are limited in usefulness.

Administration of Interferon α2a, which has some anti-angiogenic activity (Fung, 1991; Guyer *et al*, 1992 and Engler *et al*, 1994) and transplantation of retinal pigment epithelial (RPE) cells (Algvere *et al*, 1994) have also proved to be of limited usefulness, and initial promising results obtained with small groups of patients have not been confirmed in larger trials.

In addition to laser photocoagulation which, as described above, suffers from various disadvantages, the other main method of treating diabetic retinopathy is the control of blood glucose and blood pressure. The efficacy of such forms of treatment is limited by the motivation and compliance of the patient involved.

About 30% of the population above age 75 suffers from macular degeneration, and about 3 in 1000 individuals suffer from diabetic retinopathy. As each of these numbers will increase due to the aging of the population, and the increase in incidence of diabetes, there is a need for a more effective manner of treating these and other ocular diseases mediated by neovascularisation.

MECHANISM OF NEOVASCULARIZATION

Vascular endothelial cell growth factor (VEGF) is a dimeric, disulphide-bridged glycoprotein which is well-known to be synthesised and secreted by a variety of normal as well as tumour cells. Recent observations indicate that VEGF is frequently detected in the neovascular retinal membranes of patients with diabetes (Malecaze *et al*, 1994), the ocular fluid from patients with either diabetic

retinopathy or with central retinal vein occlusion (Aiello *et al*, 1994). More recently, it was found that VEGF expression was induced in conditions such as central vein occlusion, retinal detachment and intraocular tumours. In a rabbit model, levels of VEGF mRNA were elevated in the hypoxic region of the retina following induction of retinal vein occlusion. (Pe'er *et al*, 1995). Stimulation of VEGF expression by hypoxia has also been observed in other animal models (Pierce *et al*, 1995; Miller *et al*, 1994), and *in vitro* in all types of cell cultures (Simorre-Pinatel *et al*, 1994; Hata *et al*, 1995 and Thiema *et al*, 1995).

ANTI-SENSE DNA AND GENE THERAPY

The suppression of expression of genes encoding proteins which mediate undesirable activity has been achieved in a variety of situations by the introduction of 'anti-sense' DNA sequences into the DNA of target cells. These anti-sense sequences are DNA sequences which, when transcribed, results in synthesis of RNA whose sequence is antiparallel to the sequence encoding the protein. Such anti-sense sequences have been tested in a number of viral diseases. Alternatively, anti-sense oligodeoxynucleotides can be introduced into target cells; such short sequences are not themselves transcribed, but inhibit transcription and/or subsequent translation of the corresponding sense DNA sequence in the target cell.

In Australian Patent Application No. 75168/94 (HYBRIDON INC), it was shown that *in vitro* expression of murine VEGF could be inhibited in COS-1 or NB41 cells by incubation with 19- to 21-mer anti-sense oligonucleotides based on murine VEGF. A 21-mer antisense nucleotide targeted against the translational stop site was shown to be the effective sequence. There is no disclosure or suggestion of specific targeting of sequences to any tissue in the eye, or of treatment of any ocular conditions other than diabetic retinopathy.

In U.S. Patent No. 5,324,654, a method of stimulating proliferation of non-malignant cells is disclosed. The method comprises the *in vitro* treatment of cells with an anti-sense nucleotide corresponding to the retinoblastoma (Rb) gene to inhibit expression of the Rb gene product, resulting in suppression of the expression of proteins which inhibit cell growth. In this way, proliferation of cells is encouraged. The proliferated cells can then be re-implanted if desired, and the cells may be genetically engineered to replace a specific gene prior to re-implantation. However, there is no reference to use of this anti-sense sequence to treat conditions of the eye. The invention of US5324654 is directed to establishing cell lines capable of long-term proliferation and to treatment of conditions such as muscular dystrophy and diabetes, caused by failure to express a gene.

However, successful use of anti-sense nucleotides to counter expression of a gene *in vivo* is limited by factors such as the need for co-suppression of mutant gene expression (Milan, 1993; McInnes and Bascom, 1992), or the need for high concentrations of the anti-sense nucleotides (Akhtar and Ivinson, 1993).

Attempts to increase uptake of anti-sense sequences into the target cell by encapsulating these sequences in liposomes have been largely unsuccessful.

Another manner in which the targeting may be achieved is the employment of virus-mediated DNA transfer, using viruses such as the Sendai virus. Sendai virus is an RNA virus which has been shown to deliver DNA and proteins into cells with more than 95% efficiency (Kaneda *et al*, 1987). In this gene transfer system, DNA nuclear protein complex in liposomes is directly introduced into the cytoplasm of the cell by the fusion activity of Sendai virus. The DNA is delivered rapidly into the nucleus with nuclear protein. Sendai virus-mediated gene transfer occurs by fusion of the virus with the cell membrane, and

bypasses the endocytic pathway. Recently, highly efficient delivery of anti-sens or plasmid DNA into target cells by Sendai virus have been observed. Both the anti-sense and plasmid DNAs retained their activity not only in culture but also *in vivo* (Kaneda *et al*, 1987). However, the use of this virus is limited by the fact that there are no suitable constructs available at present to use as vectors. In addition, the transferred DNA can only be expressed for a limited period of time since the gene transfer is mediated by fusion.

Retroviruses have been widely used for somatic tissue gene therapy (Boris-Lawrie and Temin, 1993). They can target and infect a wide variety of host cells with high efficiency, and the transgene DNA integrates into the host genome. Theoretically, the integration of the DNA will provide permanent production of the transgene which could result in permanent rescue of the cells. However, retroviruses cannot infect non-dividing cells (Salmons and Günzburg, 1993). Furthermore, the retrovirus particles ar unstable *in vivo*, which makes it difficult to achieve high virus titre with inoculation. In addition, there are significant concerns regarding the oncogenicity of the integrated viruses. The inability of retroviruses to infect non-dividing cells means that they cannot be selected as candidates for gene transfer in the eye, as the most important target cells such as photoreceptors and RPE cells are non-dividing cells.

The usefulness of herpes simplex virus vectors has been limited by their poor efficiency of infection (Culver *et al*, 1992). Two types of vectors have been developed, namely the replication defective recombinants and the plasmid-derived amplicons. The latter requires a helper virus. Although the toxic genes can be removed from the herpes simplex virus with difficulty, the constructs remain cytotoxic (Johnson *et al*, 1992). In addition, th long term expression of the s quenc s ins rted has be n

unsuccessful to date, and there are problems with the regulation and stability of the constructs. Th application of modified herpes simplex viruses to the eye in gene therapy poses major concerns because of their pathogenicity. Herpes zoster virus infection causes serious infections in the eye, frequently resulting in blindness requiring corneal transplantation.

Adenoviruses have been widely used for gene transfer in both non-dividing and proliferating cells. They can accommodate DNA up to 7.5 kb, and provide efficient transfection and high viral titre. The main advantage of using these rather than retroviruses is the ability to infect a wide range of non-dividing target cells (Kozarsky and Wilson, 1993). Replication-defective adenoviruses are considered to be relatively safe, in that these viruses are common pathogen in humans, usually causing relatively benign conditions such as colds. The vectors carry tumour genes with a deletion mutation, lowering the possibility of becoming oncogenic (Siegfried, 1993). In the first experimental gene therapy trial approved by the US National Institutes of Health Recombinant DNA Advisory Committee, recombinant adenoviruses were used to treat individuals suffering from cystic fibrosis.

However, the main disadvantage of adenoviruses is their transient gene expression. This is a result of the lack of integration of the transgene into the cellular genome. Furthermore, few attempts at gene delivery to non-dividing cells have been successful. The first successful gene transfer into the brain, which consists of non-dividing cells, was reported in 1993 using adenoviruses (Le Gal La Salle *et al*, 1993).

The targetting of a specific gene to a specific cell has not been attempted, and no one ocular type has been singled out. Specific targetting using adenovirus alone is expected to be difficult, as the virus has the

ability to transfect a larg variety of cell types. For treatment of ocular diseases, in which other sites in the body are largely or entirely unaffected, it is highly desirable to deliver the therapeutic agent selectively to the target tissue in the eye. For anti-sense DNA, it is essential that the DNA be actually taken into these target cells.

These advances in gene therapy have led to further studies of the delivery and expression of transgenes into target cells, such as β-galactosidase transgene into the retina (Bennett *et al*, 1994, Li *et al*, 1994 and Mashmour *et al*, 1994) using recombinant adenovirus as a delivery system. High levels of transgene expression within 3 days in the RPE layer and within two weeks in the photoreceptor cells of the neural retina in young animals were observed. The expression of the reporter gene was followed up to 9 weeks. In older animals, neither subretinal nor intravitreal injections induced the expression of the β-galactosidase transgene in the photoreceptor cells (Li *et al*, 1994).

Australian Patent Application No. 61444/94 shows the uptake of replication-defective recombinant adenovirus by various tissues in the eye following injection into the anterior chamber, the vitreous humour, or the retrobulbar space, and that the reporter gene β-galactosidase is expressed. However, this document does not show that such forms of viruses successfully incorporate the active agent into the target cell or area. Nor is there any disclosure or suggestion that VEGF can be used to heal any ocular condition.

Another known obstacle to success of using anti-sense nucleotides as a form of therapy for the eye is the inability of the nucleotide to enter the target cells, and the limited stability of the oligonucleotides which have been modifi d, eg. phosphothioate oligonucleotides (Helene 1991). These factors greatly restrict the success of gene

therapy *in vivo*, particularly in the long term. In the treatment of retinal dis ases, the ability to delay progression of the conditions by about 12 months would greatly increase the value and effectiveness of long term therapy.

When using adenoviruses as a transport vector for retinal gene therapy, the associated cytotoxicity has been shown to be dose-dependent (Mashmour, 1994) and poses another difficulty in using such a vector. In order to decrease the dose of a given vector but retain its transfer efficiency, an adjuvant may be used. Adjuvants such as lipofectin have been shown to increase the uptake of "naked" DNA by cells.

Hyaluronic acid (HA) is a large, complex oligosaccharide consisting of up to 50 000 pairs of the basic disaccharide glucuronic acid-β(1-3) N-acetylglucosamine β(1-4). It is found *in vivo* as a major component of the extracellular matrix. Its tertiary structure is a random coil of about 50 nm in diameter. It has the ability to bind a large amount of water, which *in vivo* makes it a viscous hydrated gel with viscoelastic properties. It is found in this form in the mammalian eye, both in the vitreous and in the extracellular matrix.

The important characteristic of HA, in terms of its acting as a potential adjuvant is its ability simultaneously to bind to other molecules and to bind to cell membranes. Cell surface receptors specific to HA have been identified, including the histocompatibility antigen CD44 receptor for hyaluronan-mediated motility (RHAMM), intercellular adhesion factor (ICAM), and some homologous proteins in the CD44 family.

The putative adjuvant mechanism of action is based on the the presence of surface receptors on many cell types and the ability of HA to bind molecules. The binding of virus to th cell membrane facilitated by HA would allow the usual ndocytotic mechanisms of viral uptake to be more

eff ctive.

Even though HA has b en wid ly us d in ey surgery as a replacement for vitreous humour lost during the surgical procedure, we are not aware of any suggestion in the art that HA promotes uptake of any pharmaceutical agent into any cells or tissues in the eye. Similarly, although HA has been suggested to promote penetration of pharmaceutical agents such as antibiotics or anti-cancer agents, as set out in Australian Patent Application No. 52274/93 by Norpharmco, this specification does not suggest that HA promotes uptake of any agent, let alone DNA, or viruses by individual cells of any type. In particular, this invention does not teach the use of HA via intra-ocular injection.

The retinal pigment epithelium (RPE) is a non-renewable single cell layer in the eye, situated between the neural retina and the choroid. The cells of the RPE are phagocytic neuroepithelial cells which form the outer most layer of the retina. The phagocytic properties of these cells have long been known, and have been reviewed (Bok and Young, 1979).

We have now found that the phagocytic nature of the RPE cells will increase the uptake of molecules such as oligonucleotides and viruses, following injection into the vitreous space *in vivo*. These RPE cells show increased uptake of virus compared to other cell types. Our findings enable the induction of both long-term and short-term inhibition of VEGF expression in retinal or choroid epithelial cells, and hence inhibition of neovascularisation of the retina.

SUMMARY OF THE INVENTION

According to one aspect, the invention relates to a pharmaceutical composition for treatment of retinal diseas s mediated by abnormal vascularisation, comprising anti-sens nucleic acid sequences directed against vascular

endothelial growth factor, and optionally further comprising adjuvants for increasing cellular uptake such as hyaluronic acid, together with a pharmaceutically acceptable carrier or vehicle.

In separate embodiments, this aspect of the invention is directed to treatment for such retinal disease in the short term (up to about two months), the long-term (up to about one year), and indefinite (for the life of the patient). In the first embodiment, for short-term treatment the invention provides one or more anti-sense oligonucleotides having 100% complimentarity to a corresponding region of the VEGF gene. The oligonucleotide should have 16 to 50 nucleotides, preferably 16 to 22, and more preferably 16 to 19 nucleotides.

For long-term inhibition, the invention provides a recombinant virus comprising VEGF DNA in the anti-sense direction. This VEGF DNA is a long sequence, which for purposes of this specification is to be understood to represent a VEGF sequence of greater than 20 nucleotides in length, preferably greater than 50 nucleotides, ranging up to the full length sequence of VEGF. In this embodiment, the recombinant virus is accumulated in RPE cells, and produces anti-sense VEGF *in situ*, thereby inhibiting VEGF expression in the RPE cell. The VEGF is most preferably human retinal pigment epithelial (RPE) or choroidal endothelial VEGF.

In another aspect, the invention provides a method of prevention or amelioration of retinal diseases caused by abnormal neovascularisation, comprising the step of administering an effective amount of an anti-sense nucleic acid sequence directed against VEGF into the eye, thereby to inhibit neovascularisation.

For indefinite inhibition, the invention provides an adeno-associated or similar virus comprising VEGF DNA in the anti-sense direction. As in the embodiment directed for long-term treatment, this VEGF DNA is of at least 20

nucleotides, preferably greater than 50 nucleotides. The adeno-associated or similar virus facilitates integration of anti-sense VEGF DNA into the RPE cell genome, thus enabling expression of anti-sense VEGF for as long as the cell remains functional. Eye diseases which may be treated using the compositions and methods of the invention include but are not limited to age-related macular degeneration (ARMD) and diabetic retinopathy. Other ocular condition and tissues in which vascularisation occurs, for example rubeosis iridis or cornea neovascularisation, may also be treated by the invention.

The anti-sense sequence may be carried in a replication defective recombinant virus, as a vector or vehicle. The vector preferably comprises replication defective adenovirus carrying promoters such as the respiratory syncytial virus (RSV), cytomegalovirus (CMV), adenovirus major late protein (MLP), VA1 pol III or β-actin promoters. In a particularly preferred embodiment, the vector is pAd.RSV, pAd.MLP, or pAd.VA1. In a more particularly preferred embodiment the virus used is Ad.RSV.αVEGF or Ad.VA1.αVEGF. The vector may also comprise a polyadenylation signal sequence such as the SV40 signal sequence.

In a preferred embodiment, human VEGF is subcloned into the vector, in order to create the restriction sites necessary for insertion, to form an adenovirus plasmid carrying VEGF or partial sequences thereof in an anti-sense direction, which can then be linearised by restriction enzyme digestion. The linearized plasmid which can then be co-transfected with a linearized replication defective adenovirus. In a suitable permissive host cell such as the kidney 293 cell line.

The compositions of the invention may be delivered into the eye by intra-vitreal or sub-retinal injection, preferably in an appropriate vehicle or carrier. Such methods of administration and vehicles or carri rs for

such injection are known in the art. Alternatively, *ex vivo* delivery of the compositions of the invention may be achieved by removal of RPE cells from the patient to be treated, culturing the cells and subjecting them to infection *in vitro* with a replication-defective adenovirus or an adeno-associated virus as defined above. RPE cells carrying the virus are then injected into the sub-retinal layer of the eye of the patient.

While the invention is specifically described with reference to conditions of the eye, the person skilled in the art will be aware that there are many other pathological conditions in which VEGF is of importance. Such a person will understand that the antisense oligonucleotides and the recombinant viruses of the invention are applicable to treatment of such other conditions.

Brief Description of the Figures

Figure 1 shows the results of GeneScan analysis of persistence of anti-sense oligonucleotides *in vivo* in the retina following a single intra-vitreal injection.

Figure 2 is a graphical representation of the number of phagosomes in the RPE layers of Long-Evans rats. Doses were as follows: Low 6.6 μg, medium 66 μg and high 132 μg of CATSC anti-sense oligonucleotide. Each column shows the mean and standard deviation of the number of phagosomes in five randomly selected areas in the rat retinas.

Figure 3 is a graphical representation of the number of phagosomes in the RPE layers of RCS-rdy+ rats. Experimental animals were injected with 66 μg of sense oligonucleotides (S1) and 66 μg of antisense oligonucleotide (CATSC).

Figure 4 shows the effect of increasing the titre of adenoviral vector on the number of cells expressing the adenoviral transgene. In all cases, the incubation period

was 16 hours. RPE7 denot s Human retinal pigment epithelial cells from a 7 year old donor; F2000C denotes F2000 fibroblastic cells. The C suffix on the F2000 key indicates that the counts for the F2000 cell expression have been corrected for direct comparison with the RPE7 cells.

Figure 5 shows the effect of increasing the time of incubation with the adenoviral vector on the number of cells expressing the adenoviral transgene. In all cases, the concentration of the adenoviral vector was $2x10^6$ p.f.u./ml. The C suffix on the F2000 key indicates that the counts for the F2000 cell expression have been corrected for direct comparison with the RPE7 cells.

Figure 6 is a graphical representation of the effect of Hyaluronic Acid (HA) on the number of RPE7 cells expressing an adenoviral transgene for a fixed viral titre. The three bars indicate the effect of 0.001% HA, 0.005% HA and no HA (control). The error bar indicates one standard deviation.

Figure 7 is a graphical representation of the effect of Hyaluronic Acid (HA) on the number of F2000 cells expressing an adenoviral transgene for a fixed viral titr . The three bars indicate the effect of 0.001%HA, 0.005%HA and no HA (control). Error bar indicates one standard deviation.

Figure 8 shows the immunofluorescent staining of HA receptors in RPE7 and F2000 fibroblasts 8a. CD44 staining on RPE7; 8b. ICAM staining on RPE7; 8c. RHAMM staining on RPE7; 8d. CD44 staining on F2000 fibroblasts; 8e. ICAM staining on F2000 fibroblasts; 8f. RHAMM staining on F2000 fibroblasts.

Description of the Invention

The invention will now be described by way of referenc only to the following non-limiting examples. In some of thes examples, the feasibility of the methods

utilised in the invention is demonstrated using anti-sense oligonucleotides complementary to cathepsin S (CATSC).

Example 1 Accumulation of Antisense Oligonucleotides in the RPE Cell Layer

Human retinal pigment epithelial cells were cultured and on the third passage were used for *in vitro* experiments. Confluent cultures were incubated with bovine rod outer segments (ROS) to mimic the *in vivo* situation. A fluorescein-labelled anti-sense oligonucleotide complementary to human cathepsin S (CATSCF) was added to the medium of these cells and after 7 days of incubation, the cells were harvested. The presence of fluorescein-labelled oligonucleotides within the RPE cells was detected by fluorocytometry (FACS). A GeneScan DNA analyser was used to assess the presence and stability of the oligonucleotides in the cells. The fluorescence of cultured RPE cells was increased by about 100-fold, demonstrating the presence of the anti-sense oligonucleotides within the RPE cells. These results are summarised in Table 1.

Table 1

Fluorocytometer measurements of human RPE cells incubated with or without complementary CATSCF

SAMPLE	FACS READINS
RPE + ROS	5.94
RPE + ROS + CATSC	8.50
RPE + ROS + CATSCF	461.50

It was not known if the fluorescence was emitted by the full length CATSC or by degraded oligonucleotides. Using GeneScan, it was demonstrated that the fluorescenc was larg ly due to a 19-mer oligonucleotide, which appeared

at a position similar to that of CATSCF. Using a similar procedure, it was observed that CATSC oligonucleotides were still intact after 7 days of incubation.

Example 2 Cellular Distribution of Oligonucleotides in Retinal Cells and Stability of Oligonucleotides Following Injection Into Eyes

One mmole of CATSCF was injected into the vitreous humour of 6-week old non-pigmented RCS-rdy$^+$ rats, and the movement of the oligonucleotides were followed by confocal fluoromicroscopy. Fluorescein (1mmole) was also injected as a control. Animals were euthanised 2 hours, 3 days and 7, 14 and 28 and 56 days after injection. Following euthanasia, the injected eyes were enucleated, frozen, sectioned and immediately used for confocal microscopy without fixation.

Two hours after intravitreal injection of CATSCF the penetration of the oligonucleotides were observed in the ganglion cell layer at 2 hours and also in the photoreceptor and pigment epithelial layers at 3 days. However, 7 days following injection, only the RPE layer had significant amounts of CATSCF. At 14, 28 and 56 days, a fluorescent signal was maintained in the RPE layer, and no signal was observed in any other cell types. These results show that a large proportion of CATSCF was taken up by the phagocytic RPE cells.

Following intravitreal injection as described above, eyes were dissected, the retina was removed, and th DNA extracted. The purified DNA was subjected to GeneScan analysis. The presence of undegraded fluorescein-labelled oligonucleotide was demonstrated in the rat retinas after 7, 14, 28 and 56 days of injection, as shown in Figure 1. The intensity of the signal had significantly diminished by 56 days.

These r sults demonstrate that following intravitreal injection, oligonucleotides accumulate in the RPE cells. The oligonucleotides are present in the RPE layer up to 30 days and remain in a biologically active form during this period of time.

Example 3 Biological Activity of Anti-Sense Oligonucleotides

Female sixty day-old pigmented rats of the Long-Evans strain were obtained from Charles River Breeding Laboratories, Wilmington, MA.

Sixty day old non-pigmented RCS-rdy + rats were obtained from our colony. The animals were acclimatised to a 12 hr light/ 12 hr dark lighting cycle, with an average illuminance of 5 lux for at least 10 days prior to experimentation.

Animals were anaesthetised by intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight). Intravitreal injections through the pars plana were made using a 32 gauge needle. The left eyes served as controls, and the right eyes were injected with 3µl of 150 mM sodium chloride (saline), or with 3 µl of saline containing 6.6, 66 or 132 µg of CATSC respectively, an anti-sense oligonucleotide described earlier (Rakoczy *et al*, 1994) or 66 µg of sense oligonucleotide S1, 100% complementary to CATSC. Injected animals were allowed to recover from anaesthesia, and at one week post-injection were sacrificed by an overdose of sodium pentobarbital and used for morphological examination. All animals were killed within half an hour at the same time of the day, approximately 4 hours after light onset. Two to three animals were used for each dose.

Following enucleation, whole eyes were immersed in 2.5% glutaraldehyde and 1% paraformaldehyde in 0.125M sodium cacodylate buffer, pH 7.35. The cornea and 1 ns were dissected free and the eyecup trimmed for orientation

purposes. The tissue was fixed overnight at 4°C and then post-fixed for 1 hour in 1% osmium t troxide at room temperature. After ethanol dehydration, the tissue was embedded in epoxy resin. Retinal sections were prepared for transmission electron microscopy as described previously (Kennedy *et al*, 1994).

Histological data were obtained by light microscopy. Semi-thin 1 μm sections were cut using a LKB 2088 Ultratome (LKB-Produkter, Sweden) with a diamond knife and stained with toluidine blue. The number of phagosomes that accumulated in the RPE cells of each specimen injected with saline, low (6.6 μg), medium (66 μg) or high 132 μg) dose of CATSC and 66 μg of S1 sense oligonucleotide was determined. From each eye, five sets of counts were made at 40 fold magnification and the standard deviation was calculated. Each set consisted of the total number of phagosomes in 250 μm length of RPE from 6 different randomly selected areas. The number of phagosomes that accumulated in the RPE of the control eyes, low medium and high doses of CATSC were analysed and graphically represented. Comparisons were made using the analysis of variance following the general linear models procedure of the SAS^R (version 6) statistical package (SAS Institute Inc., USA).

The results show that we successfully tested an anti-sense oligonucleotide (CATSC) in two strains of rats. The number of phagosome-like inclusion bodies present in control Long-Evans and RCS rdy + rats was not significantly different, 35.8+11.6 and 47.29+14.8 (mean ± SD), respectively, The intravitreal injection was non-traumatic. Light microscopic examination of the retinas of the saline injected eyes revealed no damage to the outer layers of the retina, and there was no increase in the number of phagosome-like inclusion bodies in the RPE layer when compared to the control non-injected animals. Long-Evans rats were used to identify the minimum amount of

CATSC required to induce biological changes in the RPE layer. In th control eyes and in those injected with low dose (6.6 μg) of CATSC, the number of phagosome-like inclusions within the RPE cells were 35.8+11.6 and 35.0+7.4 respectively. In animals injected with higher doses (66μg and 132 μg), the number of phagosome-like inclusions were 96.2+13.6 and 141.0+34.7, respectively, and the difference was statistically significant when compared to the control and low dose samples (Figure 2).

RCS-rdy+ rats injected with 66 μg of CATSC also demonstrated a statistically significant increase in the number of phagosome-like inclusion bodies, ie 204.20+39.3 when compared to the 47.20+14.8 in controls. In contrast, the injection of 66 μg of sense oligonucleotide (S1) did not increase the number of phagosomes (Figure 3) present in the RPE Layer, (34.4+12.54).

The inclusions found in RPEs of CATSC-injected Long-Evans and RCS-rdy+ animals were spherical in shape, and clearly distinguishable from the very dark, small elliptical melanin granules present in Long Evans rats. In the presence of 66 μg of CATSC, the tips of the outer segments showed signs of disorganisation and there were some vacuoles present in the outer nuclear layer. However these changes were not observed in S1 sense oligonucleotide-injected animals.

Electron microscopic examination of the RPE layer of a CAT SC-injected eye revealed no significant changes in the morphology of RPE cells. Melanin granules appeared smaller and less concentrated due to regional differences. Individual mitochondrial profiles were smaller in the treated group than in the controls, although the number was greater in the treated than in the untreated animals. Electron microscopic examination confirmed that the structures of the undigested material was similar to that of phagosomes. The numerous phagosomes s en in th RPE layer of rats treated with CATSC were paranuclear, and

contained mainly compacted phosholipid membranes, resembling undigested photor ceptor outer segment (POS) and confirming their photoreceptor origin. There were no other morphological changes observed in the POS layer, except for the disorganised appearance of the apices in treated animals.

Example 4 Gene Transfer to the RPE Cell Layer

The nature and dynamics of gene transfer using an adenoviral vector were examined. The effects of adjuvants on the uptake of the adenovirus was also studied.

Human RPE cultures (HRPE7) were obtained from a 7-year old Caucasian donor and prepared as described in Rakoczy *et al* (1992). Human F2000 fibroblast cells were cultured, harvested and pooled in Minimal Eagles Medium (MEM, Multicel ™ Trace Biosciences, Australia), with 10% FBS (Multiser™, Trace Biosciences) and containing 125µl gentamicin (Delta West, Bently, Australia) per 100 ml medium. One ml aliquots of the pooled cell suspension were placed into each well of a 24 well plate, to ensure equal seeding of wells. Experiments were carried out with cells at confluence, and at least two parallel sets of each experimental points were obtained.

Expression of Adenoviral Transgene

Replication-deficient Adenovirus 5 carrying a RSV promoter and β-Galactosidase gene (Ad.RSV.βgal)(Stratford-Perricaudet) was cultured and purified as described by Graham and Prevac, 1991. Ad.RSV.βgal was added to each well as a 1 ml aliquot, in MEM, at a concentration of $4x10^6$ p.f.u./ml. for the time-based trials, giving a final concentration of $2x10^6$ p.f.u./ml. For the titre-based trials, concentrations of $8x10^3$, $4x10^4$, $8x10^4$, $2.4x10^5$, $4x10^5$ p.f.u./ml were added to the wells in a 1 ml aliquot, making the total volume 2 ml in each well (the final viral concentration is half of that added). All of the trials

examining the effect of increasing viral titres involved incubation of the culture with the viral suspension for a fixed period of 16 hours.

Experiments were terminated by removing the medium from each well and fixing the cells with 0.5 ml of 0.5% glutaraldehyde. The glutaraldehyde was removed after 5 minutes and the cells washed once with Phosphate Buffered Saline (PBS). Following this, 0.5 ml of X-gal stain [For 1 ml of solution (concentration in final solution): 25µl X-Gal (0.5mg/ml, BioRad Hercules, California), 44µl HEPES buffer (44mM), 100µl $K_4Fe(CN)_6$(3mM) 100µl $K_3Fe(CN)_6$(3mM, 100µl NaCl(15mM), 100µl $MgCl_2$(1.3mM), sterile distilled water to make 1 ml (531µl)] was added to each well and incubated overnight (about 16 hours) at room temperature.

Cell Counting

An Olympus TO41 phase contrast microscope (Olympus Optical Co Ltd, Tokyo, Japan) at a magnification of 200x was used. Counting was carried out by a single observer. A second observer then blind counted 25% of the samples as a countercheck. A counting graticule in the microscope was used to define the region for counting when averaging was used.

All cells staining positively with the X-Gal stain were counted. At low expression of transgene (< approximately 2000 cells/well), the entire plate was counted. When the cell count was higher, averaging was used. Cells were counted in five standardized regions and their average was used to calculate the total count for each well.

In the trials comparing the rate of expression in HRPE7 and F2000 fibroblasts, the figure for the number of F2000 cells expressing the gene was corrected. This correction reflects the different total cell number of each cell type in a confluent culture in a 24 well plate. The count for HRPE7 is $3x10^5$ per well and for F2000, it is

$2x10^5$ per well. The graphical figures (Figures 4 and 5) also contain corrected counts to allow direct comparison. Where there is no comparison between cell types, no alteration of the raw count is carried out.

In the titre-based trials, the profiles of expression were markedly different in terms of rate of increase and absolute expression. For HRPE7 cells, the expression rate appeared to have an exponential form, while in F2000 fibroblasts the profile was more linear. There was a widening gap in expression throughout the trial comparing titre. At higher viral titre, HRPE7 expression was an order of magnitude greater than F2000 cells. For the conditions and titres tried in this experiment there was an overall and constant increase in the number of cells expressing with increasing vector titre (Figure 4).

In the study of the effect of incubation time on the profile of transgene expression, the concentration of AdV.RSV.βgal was kept constant at $2x10^6$ p.f.u./ml. The profiles of expression of transgene in the two cell types were markedly different, both in terms of rate of increase and magnitude of number of cells expressing the gene. There was also a notable delay between the sharp increase in number of HRPE7 and F2000 fibroblasts expressing the gene. For HRPE7 cells, the upturn in expression rate occurred at 4 hours while in F2000 fibroblasts, it occurred at 24 hours. There is a "window" period between 4 and 24 hours where the HRPE7 expression is an order of magnitude greater than that of F2000 cells (Figure 5).

Example 5 — Effect of HA as an Adjuvant on the Uptake and expression of the β-gal Gene using a Viral Vector

HRPE7 and F2000 cells were aliquoted into 24 well plates. The cells were incubated as described in Example 4, and allowed to reach 95% of confluence. Solutions of 0.001% to 0.005% buffered sodium hyaluronate (HA) (1%

Hyaluronic acid from roost r comb; HEALON, Pharmacia AB, Uppsala, Sweden) were prepared with MEM. A dose of 10 µl of viral solution at a concentration of 4 x 10^6 p.f.u. was added to 10 ml of each of the diluted HA solutions and 10 ml of MEM for the control, and incubated for 30 minutes at 25°C with intermittent gentle shaking. To separate wells of the 24 well plate, 1 ml of each of the test and control solutions was added. There were four parallel samples for each test concentration and for the control, which were counted and averaged.

The viral/HA solutions were incubated with the cell cultures for 16 hours. Each experiment was terminated according to the procedure given in Example 4.

Table 2

Experiment 1: Expression in HRPE 7 Cells

	1	2	3	4	Mean
RPE 7/HA (0.001%)	17114	20776	18730	17998	19168
RPE7/HA (0.005%)	17688	22186	20258	22236	20592
RPE 7/Cont	10782	15480	16326	15266	14705

The mean number of HRPE7 cells expressing the transgene in each well for adenovirus alone was 14 705 (SD±2228). For adenovirus with 0.001% HA the mean number of expressing cells was 19 168 per well (SD 1561) and for 0.005% HA the mean was 20592 (SD 2143) (Figure 6). This shows an increase in number of cells expressing the transgene of 30.4% for 0.001% HA and of 40.0% with 0.005% HA.

The t test probability of the significance of the increase in number of HRPE7 cells expressing the gene, when

0.005% HA is used, compared with the control, is 0.0097, which shows a 1 vel of significance of $p<0.01$. The significance reflects the large difference between the means (20592(test) v 14705(control)) and the separation of the means by more than two standard deviations.

The t test probability of the significance of the increase in number of RPE7 cells expressing the gene, when 0.001% HA is used compared with the control, is 0.02931, which shows a level of significance of $p<0.05$. The reduced significance reflects the smaller difference between the means (19168(test) v 14705(control)).

Table 3

Experiment 2: Expression in F2000 Cells.

	1	2	3	4	Mean
F 2000/HA (0.001%)	4358	4620	4195	NA	4391
F 2000/HA (0.005%)	4506	3914	4759	4332	4378
F2000 Cont	3844	3652	3875	3748	3780

The protocols for examining the effect of HA on the expression of a transgene in F2000 fibroblasts were the same as that for HRPE7. The numbers of cells expressing transgenes were significantly less than for HRPE7, which is consistent with the results demonstrated in Example 4. The mean number of cells expressing in each well for adenovirus alone was 3780 (SD±100). For adenovirus with 0.001% HA, the mean number of expressing cells was 4391 per well (SD±214) and for 0.005% HA the mean was 4378 (SD355)(Fig. 7.). This shows an increase of 15.8% for 0.001% HA and of 15.5% with 0.005% HA in the number of cells expressing the adenoviral transgene.

The two-tailed student *t* Test was used to assess the significance of the difference between the means for each set of experimental data. For each experiment, the means, the Standard error of the differences of the means and the p value for the t Test are given. In both experiments, HA gave very significantly increased uptake ($p < 0.05$).

The t test probability of the significance of the increase in number of cells expressing transgene for the F2000 fibroblasts with 0.005% HA, compared with the control, is 0.0044, which shows a level of significance of $p<0.01$. The high significance here reflects the large difference between the means (4391(test) v 3790(control)) and the small variation within the two samples. The standard deviation is 214(test) and 111(control).

The t test probability of the significance of the increase in number of cells expressing transgene for the F2000 fibroblasts with 0.001% HA, compared with the control, is 0.0195, which shows a level of significance of $p<0.05$. There is a greater variation in the raw figures, and the standard deviation is higher than for the 0.005% sample (355 v 214), which accounts for the higher p value.

Preliminary trials of chondroitin sulphate and lipofectamine as adjuvants were also carried out in order to assess the likely efficacy. These agents had no significant effect on gene expression in HRPE7 cells.

The following doses of adjuvants were also used:-

Table 4

HA Concentration

Amount of viral solution	0.05%	0.01%	0.005%	0.001%	Control	Control
5 µl	176[a]	318	319	316	279	282
10 µl	305[a]	906	802	645	623	609
25 µl	-[a]	714[b]	1682	1822	1478	1184
50 µl	-[a]	2772	2692	3328	2250	1822

The figures represent the effect of HA concentration on the uptake and expression of β-gal transgene. Increasing virus concentration resulted in an increase in the number of β-gal expressing cells. The numbers represent the number of RPE cells staining positive for β-gal following 16 hours incubation of virus in the presence of HA in a 24 well plate (cc 2x10⁶ pfu/ml).

[a] The viscosity of these solutions precluded adequate dispersion of the HA and made them very difficult to manipulate.

[b] It was not clear why this figure fell so far outside of the normal distribution of the other results.

Example 6. Demonstration of HA Receptors on the cell membrane of HRPE7 and F2000.

Polyclonal RHAMM (Receptor for Hyaluronan Mediated Motility) antibodies were kindly provided by Dr E Turley, Manitoba Institute of Cell Biology, Canada. The antibody was used at a dilution of 1:75. Monoclonal InterCellular Adhesion Molecule 1 (ICAM-1) antibodies (Boehringer-Mannheim) were used at a concentration of

4µg/ml and monoclonal homing receptor CD44 antibody (CD44) was used at a concentration of 4µg/ml (Boehringer Mannheim Biochemica, Germany). Monoclonal anti-human IgG antibody and rat non-immune serum were kindly provided by Dr M. Baines, Lions Eye Institute, Perth, Australia. They were used at a concentration of 4µg/ml and a dilution of 1:75 respectively. Anti-Mouse IgG (Fab specific)-FITC conjugate secondary antibody was used at a 1:64 dilution and anti-Rabbit IgG (whole molecule)-FITC conjugate secondary antibody was used at a 1:100 dilution (Sigma Immunochemicals, St Louis, Missouri).

HRPE7 and F2000 fibroblast cells were cultured in Lab Tek 8-well slide chambers (Nunc Inc. Naperville, Illinois). Cell cultures were fixed with methanol at -20°C for 10 minutes before immunofluorescent staining. All primary antibody solutions were incubated for 1 hour. The primary antibodies used for each of the two cell types were monoclonal anti ICAM-1, anti-CD44 as test and monoclonal anti-Human IgG as control, and polyclonal anti-RHAMM with a non-immune rabbit serum as control. Following the removal of the primary antibody, each well was washed three times with PBS and the secondary antibody was applied for 1 hour. The secondary antibody to the monoclonal antibodies was antimouse IgG and the polyclonal was anti-rabbit IgG. The secondary antibodies were applied to tissue without primary antibody as a further control. Finally, on removal of the secondary antibody, each well was washed a further three times before the well chambers were removed and the slides mounted with Immuno Fluore Mounting Medium (ICN Biomedicals Inc, Aurora, Ohio).

Immunohistochemical staining for CD44 using a monoclonal antibody demonstrated positive staining for both HRPE7 cells and F2000 fibroblasts, as shown in Figures 8a and 8b respectively. The staining had a distribution consist nt with the cell surface, as the staining pattern was the same as the cellular outline of cultured tissue.

A monoclonal human anti-IgG was used as control, and was negativ for both HRPE7 and F2000 fibroblasts. A second control, using secondary fluorescent antibody with no primary antibody was also negative for both cell types.

Immunohistochemical staining using a monoclonal antibody for ICAM-1 demonstrated positive staining for both HRPE7, and F2000 fibroblasts, as shown in Figures 8c and 8d respectively. The staining had a similar distribution to that of CD44, but the signal was slightly weaker. The same controls as for CD44 were used for ICAM-1 staining, and were also negative.

Staining for RHAMM receptors using a rabbit polyclonal antibody was positive for both HRPE7 and F2000 fibroblasts, as shown in Figures 8e and 8f respectively. The distribution of staining, however, was markedly different in the two cell types. In HRPE cells the staining pattern was predominantly nuclear, with a very faint cytoplasmic outline (Figure 8e). The distribution of staining in F2000 fibroblasts was similar to that of CD44 and ICAM-1, with no significant nuclear signal observable over the cytoplasmic or cell outline pattern.

The control serum was a rabbit non-immune serum, which was negative for HRPE7 but gave a very weak signal in F2000 fibroblasts. In both cases, the secondary fluorescent antibody alone did not lead to a positive signal from either cell type.

Example 7 Up and Down Regulation of Cathepsin D Expression in NIH 3T3 Cells

A 1620 bp HindIII fragment of human cathepsin D was subcloned into pHBApr-1-neo vector in both sense and anti-sense directions. Positive clones were selected, and the orientation of the fragments was confirmed by EcoRI restriction enzyme analysis. For the transfections of NIH 3T3 cells the clones carrying cathepsin D in the anti-sense and sense directions were on caesium chloride density

gradients.

NIH 3T3 cells were seeded on to 6-well tissues culture plates at a concentration of $2X10^5$ in 2 ml DMEM supplemented with 10% fetal bovine serum (FBS). The cells were incubated overnight at 37°C until they became 70% confluent. Having reached confluency, the cells were washed twice with serum and antibiotic-free medium. Lipofection reagent (10 µl) (GIBCO-BRL) diluted in 100 µl of OPTI-MEM (GIBCO-BRL) were gently mixed and incubated at room temperature for 15 minutes. Following incubation, an additional 800 µl of OPTI-MEM was added to the mixture. This diluted mixture was gently overlaid onto the washed NIH 3T3 cells. The cells were incubated for 16-20 hrs before the transfection media was removed and replaced with DMEM supplemented with 10% FBS. After a further 48 hrs incubation the cells were trypsinised and subcultured at 1:5 in media containing 10% FBS and Geneticin 418 (GIBCO-BRL) at 1 ng/ml concentration. Successfully transfected cells selected with Geneticin 418 were maintained in media supplemented with FBS and Geneticin 418 as described above. Confluent transformed cultures were frozen for storage and subcultured for further analysis. The presence of cathepsin D in the transformed NIH 3T3 cells was detected with polyclonal antibody against cathepsin D, using a conventional cytochemical technique and an alkaline phosphatase-labelled second antibody.

The presence of cathepsin D fragment of the vector was demonstrated with HindIII digestion. Positive clones showed the presence of a 1620 kb fragment. The orientation was established by ECO RI restriction enzyme digestion, which gave two fragments at 5.7 and 5.9 kb in the case of the anti-sense orientation and 4.3 and 7.3 kb in the case of the sense orientation. All NIH 3T3 cells surviving Geneticin 418 selection carried cathepsin D clones, which are antibiotic resistant. The transformed control NIH 3T3 cells did not survive th selection

procedure. Th immunocytochemistry results suggest that NIH 3T3 cells carrying cathepsin D in th s nse direction up-regulated cathepsin D production, while those carrying cathepsin D in the anti-sense direction down regulated cathepsin D production.

Example 8 Cloning and Characterisation of Human RPE Vascular Endothelial Growth Factor (RPE-VEGF)

Human RPE cells, available in our laboratory, are grown in tissue culture. To upregulate VEGF expression, cell cultures are treated in hypoxic conditions. The upregulation of VEGF expression is monitored with immunohistochemistry. The mRNA is extracted from 10^7 RPE cells, and a cDNA library carrying all genes expressed in the RPE/choroid is established using methods known in the art.

VEGF is a highly conserved molecule which is highly homologous between different species. A murine VEGF cDNA clone, available in our laboratory, is used to screen the human RPE cDNA library in order to identify the full length human RPE-VEGF clone. Positive clones are analysed by restriction enzyme analysis and finally by DNA sequencing. Full length RPE-VEGF clones are analysed to elucidate their genomic structure (initiation sequences, start and stop codons, putative exons *etc.*).

Clones carrying the full length RPE-VEGF are analysed for the expression of VEGF protein with *in vitro* translation. The identified clones are used to derive the anti-sense molecule for insertion into the vehicle system, and for the selection of the anti-sense oligonucleotides.

Example 9 Pharmaceutical Agent for the Short-Term Inhibition of VEGF Expression

Anti-sense DNA technology enables the sequence specific inhibition of target molecules without affecting

the non-targeted functions of the cell. As described above, we have demonstrated both *in vitro* and *in vivo* that anti-sense DNA can be used effectively to inhibit the anti-sense oligonucleotide into the vitreous.

A panel of 16 to 19-mer oligonucleotides, 100% complementary to parts of the VEGF gene, is selected from the 5' and 3' ends of the DNA sequence. Sense and scrambled sequences are also used as control. Phosphothioate-protected oligonucleotides are synthesized on a DNA synthesizer and subjected to purification.

Example 10 Anti-Sense Agent for the Long-Term Inhibition of VEGF Production

Human VEGF is subcloned into the appropriate vectors in order to create the restriction sites necessary for insertion into p.Ad.RSV. The adenovirus plasmid containing the human RPE-VEGF gene in the anti-sense direction is characterised, and positives are subjected to homologous recombination.

The recombinant plasmid pAd.RSV.a.VEGF is linearised Ad.RSV.bGal recombinant virus. Confluent layers of kidney 293 cells are co-transfected with the linearised plasmids and incubated by 7 days.

Using blue-white colony selection, white colonies are picked and analysed for the presence of VEGF in the anti-sense orientation. Initially, the presence of VEGF anti-sense oligonucleotide is identified by restriction enzyme analysis followed by Northern blot analysis, looking for the expression of anti-sense VEGF RNA. Modifications of the recombinant adenovirus are performed to assess their effect on improving anti-sense VEGF DNA expression. These include removal of polyadenylation signal, change of promoter, and shortening the VEGF insert.

Example 11 Construction of a Vehicle for the Permanent Expression of Target Molecules

The vehicle described in Example 10 is suitable for long-term treatment in that it provides temporary (maximum one year) expression of the anti-sense VEGF DNA molecule, and consequent protection against neovascularisation. To achieve indefinite treatment, we use a vector system which enables the integration of VEGF in the anti-sense direction into the human genome present in RPE cells using an adeno-associated virus (AAV) vector, which means that the protection against neovascularisation can be provided for the rest of the life of the patient, as long as the RPE cells remain functional.

Adeno-associated viruses are non-pathogenic, are able to infect non-dividing cells, and have a high frequency of integration. We use AAV-2, which is a replication defective parvovirus which can readily infect other cells such as RPE cells, and integrate into the genome of the host cells. Recent characterisation has revealed that AAV-2 specifically targets the long arm of human chromosome 19.

AAV constructs use varying promoter sequences in combination with a reporter gene. The expression of the reporter gene mRNA is detected with PCR amplification or *in situ* PCR, and the integration of the reporter gene is identified by chromosomal analysis of RPE cells.

Using the appropriate restriction sites, the reporter gene is replaced by anti-sense VEGF DNA. The new construct is co-transfected with the complementing plasmid (pAAV/ad) into kidney 293 cells previously infected with adenovirus type 5 to make the rAAVaVEGF construct. The construct produced is used to infect RPE cells, and the expression of anti-sense VEGF is detected by PCR amplification.

Example 12 Model Systems for Testing Inhibition *In Vitro*

Human VEGF is cloned into COS cells to produce a culture system (VEGF-COS) in which the effective inhibition of VEGF expression can be tested. The inhibition of VEGF expression is tested by Northern and Western blot analyses and quantified by immunoassay.

The toxicity of increasing concentrations of oligonucleotides on COS cells is assessed with the trypan blue assay. The proliferation of COS cells is monitored with or without increasing concentrations of oligonucleotides. The inhibition of the expression of VEGF in controls and in cultures maintained in the presence of anti-sense oligonucleotides is monitored by Northern and Western blot analyses, immunocytochemistry and by a quantitative immunoassay.

RPE cells are cultured in hypoxic conditions and the up-regulation of VEGF expression is monitored in the presence of increasing concentrations of oligonucleotides for an extended period of time. Toxicity, proliferation assay and the monitoring of VEGF expression are performed as described above.

CEC cells are cultured in normal and hypoxic conditions with or without increasing concentration of oligonucleotides. In addition to the toxicity, proliferation assay and VEGF detection, the effect of anti-sense oligonucleotide-mediated inhibition of VEGF expression on tube formation is analysed. RPE/CEC dual cultures produced in normal and hypoxic conditions will be subjected to similar tests. The same model systems are used to assess the long-term and permanent agents of the invention.

Example 13 Inhibition of RPE-VEGF Expression with Anti-Sense Oligonucleotides, Ad.RSV.aVEGF and rAAVaVEGF *In Vivo* in Rats.

Neovascularisation can be induced using pocket implants in the choroid or the subretinal layer. One of the disadvantages of these models is that the process of neovascularisation might not follow the same biochemical steps which naturally occur in humans suffering from ARMD. To overcome these difficulties we use an animal model in which choroidal neovascularisation is induced by VEGF overexpression in the RPE cells. Using recombinant adenoviruses carrying VEGF, for example Ad.RSV.VEGF, for the *in vivo* trials all animal models described above are utilised to provide us with a wide range of information. Tests are conducted to demonstrate the expression of a VEGF expression over a period of one year. Using Northern and Western blot analysis, VEGF down-regulation is monitored and immunohistochemistry is used to demonstrate the down-regulation of VEGF expression in a cell-specific manner. Using the above described animal models, choroidal neovascularisation is monitored by histology and angiography. These models are applicable to all the embodiments of the invention.

It will be appreciated that the present invention is particularly useful in the study, treatment or prevention of age-related macular degeneration, by virtue of the successful adenoviral gene transfer to the RPE. Without wishing to be bound by any proposed mechanism for the observed advantages, the higher degree of gene expression in the HRPE7 cells, compared with the F2000 cells, may indicate the ability of RPE cells to phagocytose large molecules and hence increase the uptake of adenovirus. The level of expression of the transgene may also be increased by increasing the time of exposure or the viral titre.

Th comparison studies between the HRPE7 cells and the F2000 fibroblast show that there ar marked differences in the pattern of expression between the different cell types under the same conditions. These differences could be exploited for targeting of different cells, for example RPE. The upstroke in the time/expression curve for RPE cells (Figure 5) was at 4 hours while for F2000 cells, it was 24 hours. There is, therefore, a window during which RPE cells are taking up Ad.RSV.βgal. and expressing the transgene at a significantly higher level than F2000 fibroblasts. Transfection for periods of less than 24 hours would allow use of this window as a targeting tool (eg. virus solutions could be aspirated from subretinal blebs or the vitreous after 24 hours). The titre/expression curves (Figure 5) also show that there was a difference between the cells, with RPE cells beginning to express highly at a lower concentrations. Once again, low concentration could be used to preferentially target RPE cells. A combination of lower titres for less than 24 hours would combine the two effects and provide targeted delivery.

As shown in some of the embodiments, the present invention may also be used in conjunction with adjuvants to keep viral toxicity to a minimum by reducing the titre required to effect gene transfer and expression.

We have shown a consistent and significant adjuvant effect for adenoviral gene transfer using HA. This was the case in both phagocytic and non-phagocytic cell lines. The advantage of HA is its presence as a normal component of human vitreous and extracellular matrix, and its long history of therapeutic acceptance as a viscoelastic aid to surgery.

The important feature of HA in terms of its acting as a potential adjuvant is its ability to bind cell membranes and other molecules simultaneously. We propose that the HA molecule can bind Adenovirus and the cell

membrane at the same time, and ther fore increase the contact time or concentration of virus in th vicinity of the cell membrane using this mechanism. We have identified cell surface receptors specific to HA identified on both F2000 and RPE7, as each cell tested positive for the presence of CD44, RHAMM and ICAM-1 receptors. Interestingly, the RHAMM receptors on RPE showed a nuclear distribution, and this could account for the slightly higher adjuvant effect of HA in RPE than in F2000. Our preliminary studies of *in vivo* immuno- fluorescent staining for CD44 show no signal in the neuro-retina, suggesting that HA association of the adenovirus may also be a potential targeting mechanism for RPE *in vivo*.

It will be apparent to the person skilled in the art that while the invention has been described in the Examples, various modifications and alterations to the embodiments described herein may be made without departing from the scope of the inventive concept disclosed in this specification.

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FIGURE 1

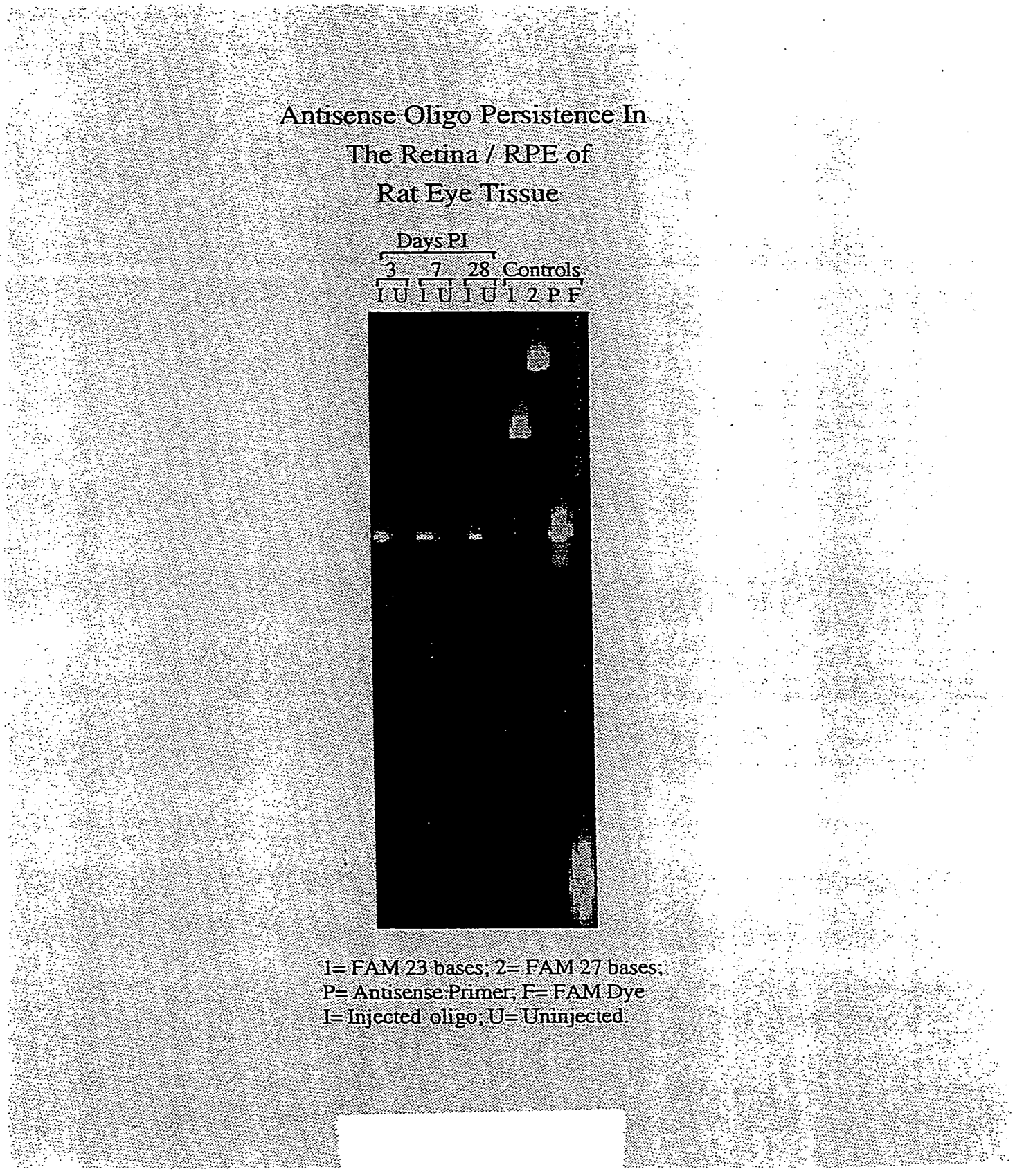
Antisense Oligo Persistence In
The Retina / RPE of
Rat Eye Tissue
Days PI
3
7
28
Controls
I U I U I U 1 2 P F
1= FAM 23 bases; 2= FAM 27 bases;
P= Antisense Primer; F= FAM Dye
I= Injected oligo; U= Uninjected

FIGURE 2

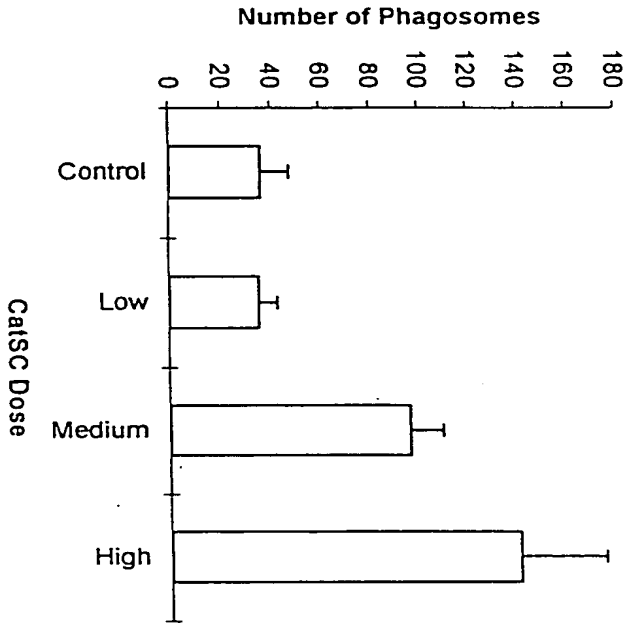
Number of Phagosomes
0
20
40
60
80
100
120
140
160
180
Control
Low
Medium
High
CatSC Dose

FIGURE 3

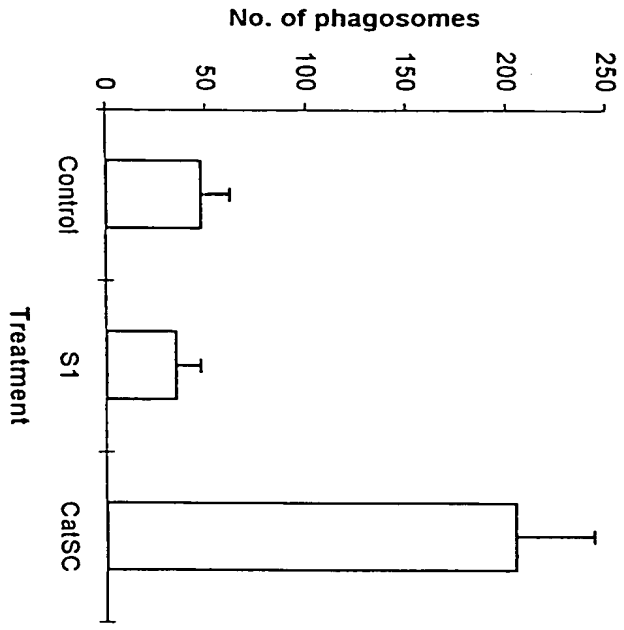
No. of phagosomes
0
50
100
150
200
250
Control
S1
CatSC
Treatment

FIGURE 4

	RPE7	F2000C
0	0	0
4	885	751
20	3357	1876
40	13232	2437
120	39721	5154
200	52564	9112

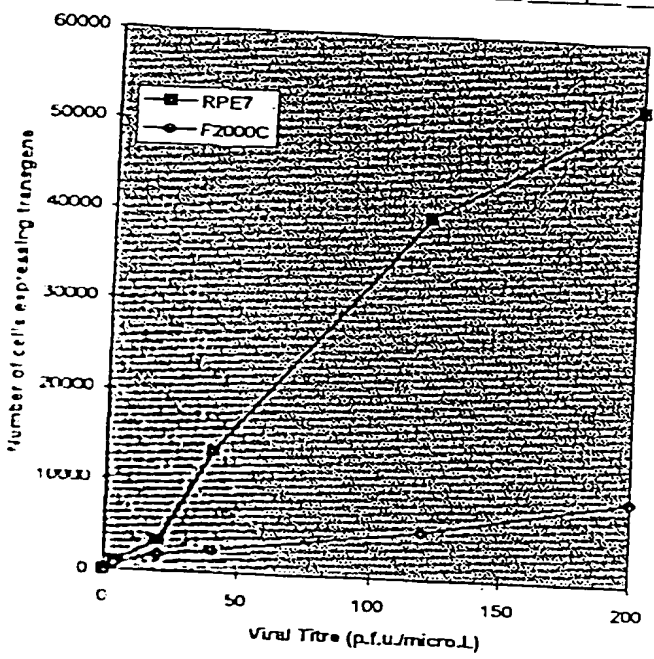

FIGURE 5

	F2000C	RPE7
0	0	0
1	186	890
2	309	1939
4	324	1860
16	2358	29248
24	5781	51453
72	126114	197934

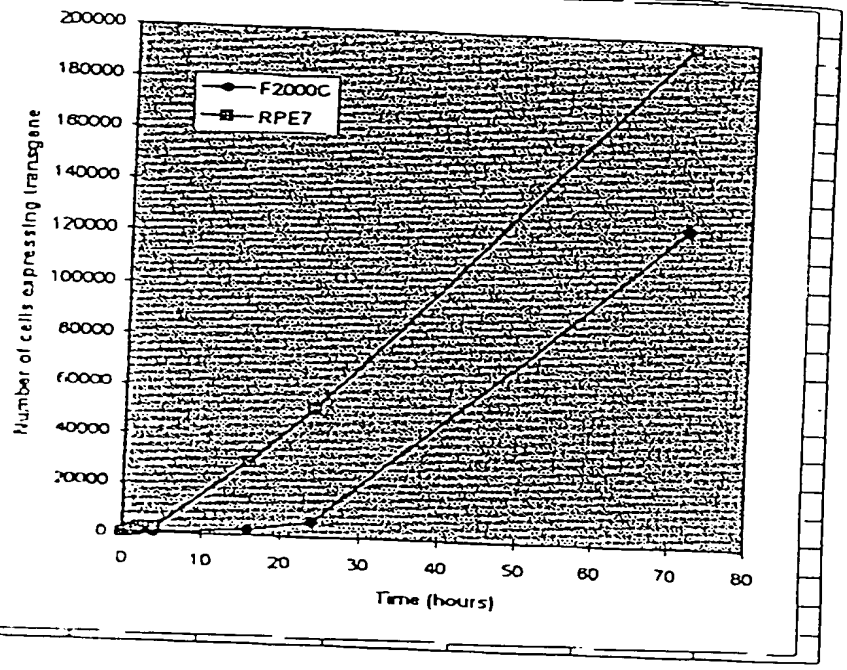

FIGURE 6

17114	17688	10782	
20776	22188	15480	
18730	20258	16326	
17998	22236	15266	
		15674	
0.001% H	0.005%	CONTROL	
19168	20592	14705.6	
1561.048	2143.855	2228.894	SD
1529.75	2100.098	1952.889	CONFID
0.011203	0.001803		T TEST

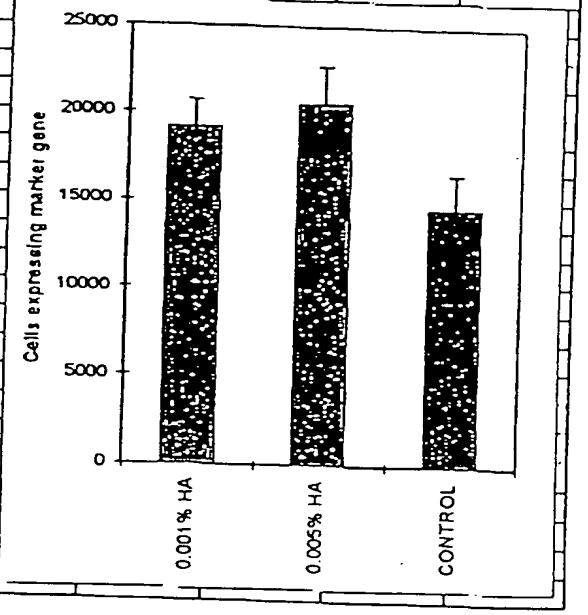

FIGURE 7

4358	4506	3844	
4620	3814	3652	
4185	4759	3875	
	4332	3748	
0.001% H	0.005%H	Control	
4391	4377.75	3779.75	
214.4132	355.4137	100.8774	SD'S
0.003727	0.008874		

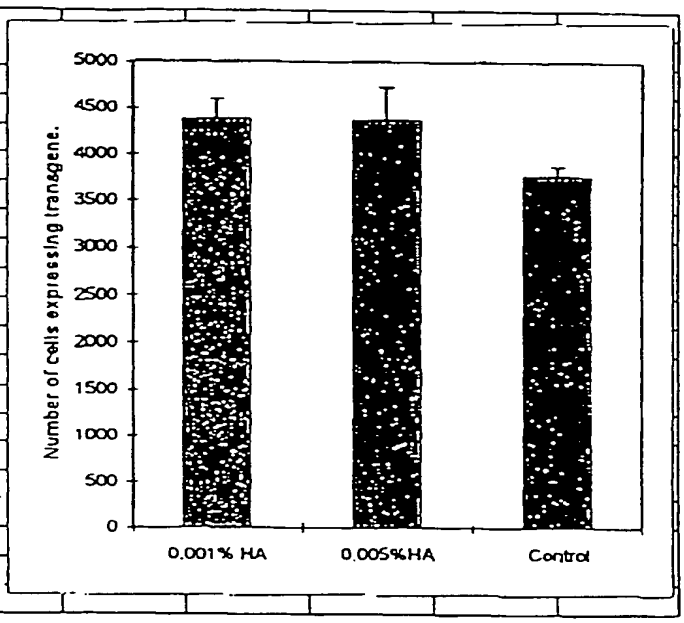

FIGURE 8